

Operant discrimination of relative frequency ratios in black-capped chickadee song

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Abstract The two-note *fee bee* song of the black-capped chickadee (*Poecile atricapillus*) is sung at many different absolute frequencies, but the relative frequencies, or “pitch ratios”, between the start and end of the *fee* note (*glissando*) and the *fee* and the *bee* notes (inter-note interval) are preserved with each pitch-shift. Ability to perceive these ratios and their relative salience varies with sex of the bird and setting: while both sexes appear to perceive changes in the inter-note interval, males appear to attend to the *glissando* in the field, and females appear to attend to both ratios. In this study, we compared directly whether male and female chickadees could discriminate between normal *fee bee* songs and songs that had one or both of the pitch ratios altered, and whether birds attended to one type of alteration over another. Both sexes learned to discriminate normal from altered songs; songs lacking an inter-note interval were more easily discriminated than songs with only the *glissando* removed. Females performed slightly better than males, including in the most difficult task with the stimuli lacking the *glissando*. Our study illustrates the value of using perceptual tasks to directly compare performance between the sexes and to demonstrate the

difference between perception of and attention to acoustic features of vocal communication.

Keywords Song perception · Operant discrimination · Black-capped chickadee · Relative frequency

Introduction

Songbirds are experts at perceiving and assessing particular aspects of song structure including amplitude (e.g. Searcy 1996), duration (e.g. Weary et al. 1990), complexity (e.g. Clayton and Prove 1989), and even note and syllable composition (e.g. Leitner and Catchpole 2002). They are particularly good—better than humans—at classifying sounds based on absolute pitch (e.g. Friedrich et al. 2007; Weisman et al. 2010). While species recognition may be partially based on absolute pitch of the vocalization (Becker 1982; Hulse and Cynx 1985; Cynx et al. 1986), relative pitch within the song also seems to be important for both species recognition and assessing quality of individuals. In several species, altering these relative pitch ratios decreases response and possibly recognition to playback compared to vocalizations with typical ratios (e.g. *Catharus fuscescens* Weary et al. 1991; *Zonotrichia albicollis* Hurly et al. 1992).

Black-capped chickadees (*Poecile atricapillus*) sing a simple, two-note song that covers a large range in absolute pitch but changes little in terms of relative pitch. Within the black-capped chickadee’s *fee bee* song, the pitch ratios between the beginning and end of the *fee* note (start of *fee*/end of *fee* = 1.056, “*glissando*”) and between the end of the *fee* note and the beginning of the *bee* note (end of *fee*/start of *bee* = 1.134, “inter-note pitch interval”) are maintained regardless of absolute pitch (Weisman et al.

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1990; see Fig. 1) and are highly conserved throughout most of the bird's breeding range (Kroodsma et al. 1999). High-ranking males, which achieve a higher reproductive success than lower-ranking ones (Otter et al. 1998; Mennill et al. 2004), are better at reliably reproducing a species-typical inter-note pitch interval (Christie et al. 2004). This raises the possibility that relative pitch cues within song may be important not just for conspecific recognition but for conveying information about the singer's quality to females and providing information for individual recognition of neighbours to males.

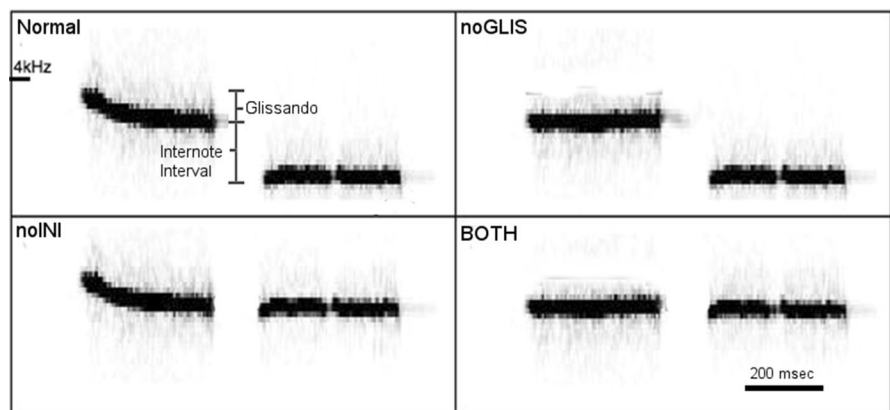
Testing the abilities of chickadees to perceive these ratios, and examining the relative importance of the two ratios to recognition, has mainly focused on the inter-note pitch interval rather than the *glissando*. Eliminating, reversing or exaggerating the *fee bee* inter-note interval reduced aggressive and territorial-type responses compared to songs with a species-typical inter-note interval in captive males (Weisman and Ratcliffe 1989), and in operant discrimination studies males were able to discriminate between songs with these types of alterations and normal songs (Weary and Weisman 1991; Njegovan and Weisman 1997). Females also can discriminate between normal songs and those with altered inter-note ratios in operant tasks (Hoeschele et al. 2012). Therefore, both male and female chickadees can perceive alterations in the species-typical interval. However, the salience of the inter-note interval as a cue seems to vary by sex. In a field playback study, males responded less strongly when the *glissando* within the *fee* note (i.e. the frequency change from the start to the end of *fee*) was altered but not when the inter-note pitch interval was changed, suggesting that territorial males attend primarily to the *glissando* portion of the *fee bee* (Shackleton et al. 1992). In contrast, estradiol-implanted females showed less sexual response to songs with either of the ratios altered, suggesting that they attend to both ratios (Ratcliffe and Otter 1996), although these results were not independent of playback order. So, although both males

and females can perceive when songs have an altered inter-note interval, in practice males appear to attend preferentially to the *glissando*, while females are sensitive to changes in both the *glissando* and the inter-note interval.

Few laboratory studies have intentionally tested perception of the *glissando* as a cue, despite the indication that both sexes attend to alterations in it. In fact, there is indirect evidence that chickadees may have difficulty perceiving the *glissando*: in a study examining whether chickadees could discriminate *fee bee* songs recorded from eastern versus western populations, chickadees that (although unintentionally) had the *glissando* available as a cue for discrimination did not use it to their advantage in solving the discrimination task (Hahn et al. 2016). In addition, male and female chickadees who heard playback of song with the *glissando* or the inter-note interval removed vocalized in response to normal and altered song, but response was diminished only when the inter-note pitch interval was absent (Roach et al. 2016). Thus, the evidence that chickadees can perceive changes in the *glissando* and its relative salience as a cue for recognition (as in Shackleton et al. 1992 and Roach et al. 2016) seem to contradict each other.

In this study, therefore, we wanted to compare directly the ability of male and female chickadees to perceive changes in the two relative pitch ratios of *fee bee* songs with one or both of the species-typical ratios removed. To do this, we used an operant training paradigm to test whether black-capped chickadees could discriminate between songs containing the two species-typical pitch ratios and songs with one or both of those ratios eliminated (i.e. reduced from 1.056 and 1.134 to 1; see Fig. 1 and Roach et al. 2016). We could test the birds' ability to perceive both the *glissando* and the inter-note interval, and determine whether one ratio was more salient the other by examining if stimuli with one alteration were better discriminated than the other. Further, we were interested in possible sex differences in perceptual abilities and relative

Fig. 1 Spectrograms showing the stimuli used: normal (N) (with species-typical pitch ratios labelled), with the *glissando* removed (noGLIS), with the inter-note interval removed (noINI), and with both alterations (BOTH)



saliency of the ratios as cues. Hoeschele et al. (2012) found that female chickadees reached a higher overall level of discrimination on their inter-note interval discrimination task, and Ratcliffe and Otter (1996) indicated that females may attend more closely to both ratios than males; if females are using accuracy of ratio production to assess male quality, they may be better than males at discerning when one or both of the ratios are absent (see Christie et al. 2004).

Based on the previous work showing birds in both the laboratory and field can perceive changes in the inter-note interval but may have difficulty perceiving the *glissando*, we expected that birds would be better at discriminating between normal and altered songs when the inter-note interval was removed (i.e. Fig. 1 noINI), compared to when the *glissando* within the *fee* note was flattened (i.e. Fig. 1 noGLIS). We also expected there to be an interaction between sex and ratio alteration. In keeping with recent operant research and evidence of the inter-note pitch interval's importance to mate choice, we expected that females would perform better than males on discriminations between songs with normal ratios and songs with the inter-note interval eliminated. However, given that males appear to attend to the *glissando* more than the inter-note interval in the field, we expected that males would be better than females at discriminating between songs with normal ratios and songs without a *glissando*.

Method

Animals and housing

Eight male and nine female adult black-capped chickadees were captured within the Halifax Regional Municipality between February 2013 and May 2014 (CWS Permit # ST2779). Capture method depended on time of year. In mid-winter, individuals were attracted using playback of chickadee vocalizations (calls and songs recorded in Ontario at least a decade before this study and mobbing calls recorded at least 20 years before this study) and captured in Potter traps baited with sunflower seeds. Later in the winter and during spring, when natural food supplies are abundant, individuals were attracted using the same playback and captured using a mist net. In all cases, likely sex was assessed at time of capture based on mass, wing chord, and tail length (Desrochers 1990) and confirmed via inspection of gonads at perfusion. Age was determined based on shape and amount of white in the outer retrices (Pyle 1997), and birds aged less than 1 year were released immediately (and not brought back to the laboratory). To allow for sufficient habituation and to minimize stress, all birds were captured before noon and transported to

Dalhousie University animal care facilities within 1 h of capture. All methods and procedures were approved by Dalhousie University's University Committee on Laboratory Animals (Protocol # 12-023).

Upon arrival to the laboratory, birds were placed in individual cages (91 cm wide × 41 cm high × 46 cm deep) made of galvanized steel mesh with a stainless steel pan for collecting waste. Each cage contained wooden perches, a swing, a water bath, and evergreen boughs. Birds had ad libitum access to drinking water, grit, and food (a mix of husked sunflower seeds and Mazuri Small Passerine diet feed, with unhusked sunflower seeds on top); food was supplemented daily with mealworms. In each case, the initial light cycle reflected natural day length. Room temperatures were maintained at approximately 18 °C.

We aimed to test all birds while they were in breeding condition in order to match the condition of birds used in previous studies examining responses to normal and altered songs (i.e. Shackleton et al. 1992; Ratcliffe and Otter 1996). Thus, birds caught during mid-winter (e.g. February) were housed for 6–8 weeks under light cycles that reflected increasing natural day lengths prior to being put in operant chambers. Birds caught later in the year (e.g. April and May), because they were already in breeding condition, were kept in animal housing for only 1 day prior to being transferred to operant chambers. We checked whether length of time in captivity prior to training affected learning by comparing statistically results between the two groups: there were no effects. After this experiment was complete, we sacrificed the birds via anaesthetic overdose and transcardial perfusion and collected brain tissue for future study.

Operant discrimination apparatus

During the experiment, each bird lived within an operant chamber and obtained all food from working in the experiment during the lights on period; day length was matched to the natural photoperiod (April/May/June) and changed weekly. The operant chamber was a modified standard cage (37 cm wide × 31 cm high × 24 cm deep) with a plastic grating floor attached to the bottom of the cage to ensure that spilled food was not accessible. Each cage was contained in a ventilated, sound-attenuating enclosure (65 cm wide × 46 cm high × 42 cm deep) lighted with a 9 W twin-tube fluorescent bulb, and contained two wooden perches, a water cup (freely available water) and a grit container. The perch in front of the feeder (the "request perch") was equipped with infrared beams to monitor when the bird landed on it to start a trial. The other, plain perch was at the same height as the request perch but farther away from the feeder; there was

approximately 20 cm between the two perches. The feeder also had infrared beams to monitor when the bird entered it (making a “go” choice) and had a motorized food cup which moved up to facilitate access to food and down to restrict access to food. The experiment was controlled by a microcomputer on each chamber and a desktop computer for each pair of chambers. Song stimuli were stored on compact discs and played via disc drives within each desktop computer (one drive for each chamber). Stimuli were played via amplifier (Azur 350a, Cambridge Audio) to a speaker (Fostex FE108E) located inside the chamber beside the feeder and were broadcast at 70 dB (measured 20 cm from the loudspeaker, i.e. the distance between the perch and the loudspeaker). See Sturdy and Weisman (2006) for a complete description of the apparatus.

Stimulus vocalizations

Sets of stimulus vocalizations for discrimination were the same as those used in a previous study (Roach et al. 2016) and consisted of *fee bee* songs of four different types (see Fig. 1): normal (species-typical frequency ratios between the start and end of the *fee* note and between the end of the *fee* and start of the *bee*); with the *glissando* removed (noGLIS; frequency ratio from start to end of *fee* note reduced to 1.0); with the inter-note interval removed (noINI; frequency ratio between end of *fee* and start of *bee* reduced to 1.0); and both manipulations (BOTH; both frequency ratio alterations). We began with unaltered (normal) recordings of six *fee bee* songs taken from a set of recordings originally made between February 1999 and May 2000 that was used in the previous work (Phillimore et al. 2003, 2011). These songs ranged in absolute frequency (measured at the beginning of the *bee* note, increasing in 50 Hz increments) from 3100 to 3350 Hz, so as to reflect the normal range of *fee bee* song frequencies (Weisman et al. 1990); the *fee* note of each stimulus began at a different frequency. Altered stimuli were prepared by eliminating one or both of the frequency ratios in each of the original songs using the audio editing program Audition 2.0 (Adobe). This gave us six stimulus quads: one normal plus three altered stimuli made from each of the original *fee bee* recordings, giving us a total of 24 song stimuli to use for discrimination training and for probe testing.

For each bird, we used a stimulus set comprised of 5 of the 6 stimulus quads (i.e. 20 of the 24 recordings) described above for training and then, when appropriate, the remaining quad for testing generalization. This meant we had six stimulus sets to use for training/testing. Assignment of sets to the birds was done semi-randomly with the aim of using each one of the stimulus quads as generalization stimuli at least once. Birds were therefore trained with five

normal stimuli, five no *glissando* stimuli, five no inter-note interval stimuli, and five stimuli with both alterations. Then, for probe testing, we added the remaining quad of stimuli to the stimulus pool to test whether the birds could generalize discrimination to an untrained example of each stimulus type.

Procedure

Preliminary training

Each bird was first trained to use the perch and feeder in order to acquire food. Initially, the bird was familiarized with the location of food: the food cup remained in the up position and, at the beginning, additional food was scattered over the top of the food cup. During this stage, a red LED light at the back of the feeder was always on. Next, the bird was trained to activate the food cup and access food by flying into the feeder mechanism only when the red light was on, after which the food cup would become available for decreasing amounts of time. Following that, the bird was trained to sit on the perch across from the feeder mechanism for 1 s in order to activate the red light, at which point it could activate the food cup by entering the mechanism. The next stage was similar, but half the time the activated red light was accompanied by a 1-s, 1000-Hz pure tone: the bird was rewarded with food access for responding to the light combined with the tone, and punished with a 30-s inter-trial interval (ITI) with the chamber lights out for responding to the light alone. In the final stage of feeder training, the red light was not used and the bird was rewarded for responding to the tone and punished for responding when the tone was absent. Completion of each of the two stages of tone/no-tone discrimination occurred when the bird discriminated the tone from no-tone contexts with a discrimination ratio (responses to tone/responses to tone + responses to no-tone) of 0.90 for two consecutive days. The bird was then trained to remain on the perch until the end of the auditory stimulus before responding. In this stage, the bird again had to sit on the perch in order to activate a tone, upon which it was rewarded with food for flying to feeder mechanism; unlike previous stages, the bird was punished for leaving its perch before the end of the tone with a 30-s ITI with the chamber lights out. This stage ensured that, during the following stages of non-differential and discrimination training, the bird would listen to the entire stimulus before responding.

Non-differential training

After learning to use the perch and feeder, each bird was exposed to the 20 unaltered and altered stimuli it would hear during discrimination training, and trained to respond

to all of them at a high and uniform rate. As in the preceding stages, the bird was required to sit on its perch for 1 s in order to activate playback of the auditory stimulus. If the bird responded by entering the feeder mechanism during the 1 s following completion of the stimulus, it was rewarded with 1 s of food access. A 30-s ITI followed. If it left the perch but did not enter the feeder mechanism, the trial ended after 1 s. If the bird did not leave the perch, the trial ended after 1 s and a 60-s ITI followed; this was intended to encourage the bird to leave the perch after each stimulus. If the bird left the perch prior to completion of the stimulus, the trial ended and the chamber lights were turned off during a 30-s ITI; this ensured that the bird was listening to the entire auditory stimulus before responding. Stimuli were selected in random order without replacement until all were played, at which point all stimuli were returned to the pool for selection; birds trained continuously for the entire day and typically completed a total of about 1500 trials per day. Each bird remained in non-differential training until they were responding to all 20 stimuli at a rate of at least 70% (number of times responding to stimulus/number of presentations of that stimulus) and were leaving the perch prior to stimulus offset on less than 10% of trials.

Discrimination training

In discrimination training, entering the feeder following a rewarded stimulus (S+) was followed by 1 s of food access. Entering the feeder after a non-rewarded stimulus (S–) was followed by a 30-s ITI with the chamber lights out, and therefore the correct response after hearing an S– was to leave the request perch and then return to start a new trial. The contingencies for other scenarios that were in place during non-differential training also applied during discrimination training. Stimuli were selected in random order without replacement until all stimuli were played, at which point all stimuli were returned to the stimulus pool and the process repeated. Birds typically completed about 1500 trials per day.

For birds in the category discrimination group ($N = 6$ males, 6 Females), the five normal song stimuli in a particular bird's stimulus set were S+s, while the 15 altered song stimuli (five each of noGLIS, noINI, and BOTH) were S–s. For birds in the pseudocategory discrimination group ($N = 3$ males, 2 females), five stimuli (from the pool of both normal and altered) were selected randomly to be S+s and the rest were S–s; a different five S+s were chosen for each bird. However, we ensured that there was at least one of each of the stimulus types (normal, noGLIS, noINI, BOTH) assigned as S+s. We asked whether birds in the category group learned the discrimination faster than birds in the pseudocategory group, indicating they were using the categories of each

stimulus type (normal, noGLIS, FI, BOTH) to afford an advantage, rather than memorizing each individual stimulus as the pseudocategory birds are forced to do.

Probe testing

The category group received 1 day of probe training after reaching criterion in order to ensure that each bird was discriminating based on the use of categories based on the pitch-related rules rather than memorization of individual stimuli. Probe testing was preceded by 2 days of modified discrimination training, during which S+ stimuli were rewarded on 85% of correct visits to the feeder rather than 100% in order to reduce the birds' ability to discriminate probe stimuli from training stimuli based on how often they were rewarded. During probe training, the stimulus pool consisted of the same S+ and S– stimuli as in training, as well as the stimulus quad (one each of normal, noGLIS, noINI, and BOTH) not initially included in each particular bird's training set. While feeder visits to the S+ stimuli from discrimination training were again rewarded 85% of the time, all four of the new stimuli were rewarded on 15% of correct visits. We did this so that birds could not discriminate the new stimuli based on reward frequency and to ensure responses to the probe stimuli were due to transfer of rules learned during discrimination training rather than new learning during probe testing. In addition, having the probe stimuli rewarded at a low and uniform rate encouraged birds to continue responding to the new songs. This procedure has been used previously in the literature using the same apparatus and design (e.g. Phillmore et al. 2003; Bloomfield et al. 2008). The contingencies in place for S+ and S– stimuli during discrimination training continued during probe testing. The 24 stimuli were selected and played in random order without replacement. Birds typically completed 1000–2000 trials during probe testing; the first 1000 trials of each bird were used in analyses.

Normal versus flat fee training

In order to further explore an interesting trend that emerged in the data from the category discrimination group, birds in the pseudocategory group underwent an additional round of discrimination training. In it, birds were presented with 12 stimuli: six normal (S+) and six without a *glissando* (S–). We did this to force the birds to attend to the noGLIS stimuli in the discrimination to determine if they were simply ignoring them when there were other S– types, or if stimuli with only the *glissando* removed made the discrimination particularly difficult. The contingencies present in the previously described discrimination training were also in place here. Stimuli were again selected in random

order without replacement. We used the pseudocategory group for this because they had not received training consistent with stimulus category but were already familiar with the stimuli and the apparatus.

Response measures and analyses

Data were collected in blocks of 500 trials, but for analyses, trials were combined into blocks of 1000 trials. For discrimination training, we calculated an overall discrimination ratio for each 1000-trial block, but used percentages of response since we had different numbers of S+ and S− stimuli. We first calculated the percentage of response to each stimulus (number of times bird flew to feeder/number of times bird flew to feeder + number of times bird left the perch and did not enter feeder). We then took the percentage of responses to S+ stimuli and divided it by the sum of the average percentages of response to both S+ and S− stimuli to compute the discrimination ratio. A discrimination ratio of 0.50 would represent birds responding equally to S+ and S− stimuli (i.e. responding at chance levels and not discriminating), while a ratio of 1.00 would indicate perfect discrimination (responding only to S+ stimuli). For category birds, individual discrimination ratios were also calculated with respect to each type of altered (S−) stimulus (noGLIS, noINI, and BOTH); for example, the discrimination ratio for noGLIS stimuli was calculated by dividing the number of feeder visits in response to S+ stimuli by the total number of feeder visits to both S+ and noGLIS S− stimuli.

For each block of trials, we also calculated how many of the five S+ stimuli were being successfully discriminated from the S− stimuli in order to make sure that birds were attending to and learning all S+ stimuli rather than generating a high discrimination ratio by responding to only one or two S+ stimuli. To do this, we calculated the response rate (number of visits to the feeder divided by the total number of times leaving the perch) for each stimulus and then used those numbers to determine how many of the S+ stimuli had a response rate higher than the upper bound of the 95% confidence interval (i.e. mean response rate plus two standard deviations) of the response rates for the S− stimuli (as in Sokal and Rohlf 1981). As with discrimination ratio, this was calculated both overall and separately for each type of altered stimulus (noGLIS, FI, and BOTH).

Both discrimination ratio and the number of S+ stimuli successfully discriminated were calculated across training, including the final block which represented the point at which the bird achieved criterion (three consecutive 500-trial blocks with an overall discrimination ratio of at least 0.90) or, if that criterion was not reached, the last block completed during discrimination training. In the case of each measure, data were used to conduct repeated

measures analyses of variance (ANOVAs). All statistical analyses were conducted in IBM SPSS Statistics 22 (Windows). Discrimination data were assessed in terms of 1000-trial blocks. When data failed Mauchly's Test of Sphericity, a Greenhouse-Geisser (GG) correction was applied to ANOVA results.

Results

Category versus pseudocategory discrimination

Discrimination ratio

Figure 2a shows the overall discrimination ratios of male and female chickadees for the first sixteen 1000-trial blocks and on the final day of training for all S+ versus all S− stimuli in the category and pseudocategory discrimination groups. We used the first 16 trial blocks as this was the minimum number of blocks we had for all birds. Among the birds in the category group, the criterion of three consecutive days with a discrimination ratio of at least 0.90

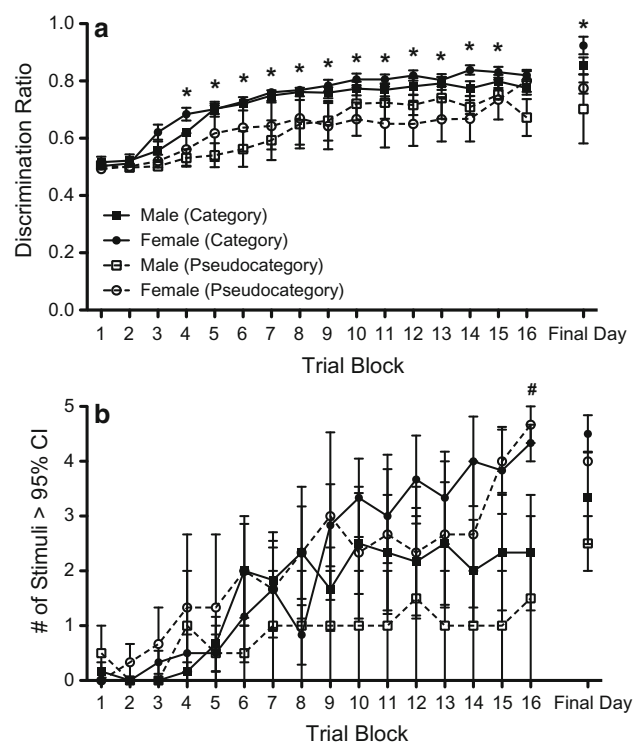


Fig. 2 Discrimination ratios (a) and number of S+ stimuli successfully discriminated from S− (b) by category and pseudocategory birds during discrimination training. Error bars represent standard errors of the means. Asterisks (*) indicate trial blocks in which performance of the category and pseudocategory groups was significantly different ($P < 0.05$). Hashtags (#) indicates trial blocks in which performance of males and females was significantly different ($P < 0.05$)

was reached by two of six males (reached after 23 and 28 1000-trial blocks) and three of the six females (after 18, 23, and 24 1000-trial blocks); the birds that did not reach the 0.90 criterion completed an average of 24.86 ($sd = 5.34$) blocks after 15 days of training. However, even birds that did not reach criterion had a minimum final discrimination ratio of 0.77 for males and 0.78 for females so were discriminating well above chance. Birds in the pseudocategory group ($N = 5$), none of which reached the criterion, completed an average of 27.4 ($sd = 12.24$) blocks after 15 days of training. The final discrimination ratios varied (0.82 and 0.58 for males, 0.74, 0.80, 0.79 for females); most were well above chance, and the male with 0.58 on the final day of training did discriminate above chance during training (highest DR = 0.81).

A sex \times discrimination group \times trial block mixed ANOVA showed a main effect of block ($F(16,208) = 46.09$, $P < 0.001$), a main effect of discrimination group ($F(1,13) = 14.93$, $P = 0.002$), but no main effect of sex ($F < 1$). A significant discrimination group \times block interaction ($F(16,208) = 1.89$, $P = 0.02$) led us to conduct separate one-way ANOVAs on trial blocks for each discrimination group. No other interactions were significant.

For each discrimination group, there was a main effect of trial block (category $F(16,176) = 72.42$, $P < 0.001$; pseudocategory $F(16,48) = 5.44$, $P < 0.001$), demonstrating that performance within each discrimination group improved over the course of training. Post hoc comparisons ($P < 0.05$) of discrimination ratios between consecutive blocks revealed that birds in the category group began improving almost immediately: their discrimination ratios improved significantly from one block to the next from the second to the fifth blocks; performance also improved between block 6 and 7 and between block 16 and the final block. In contrast, the pseudocategory group only showed such block-to-block improvements between blocks 6 and 7 and blocks 7 and 8. Independent *t* tests to compare performance between the category versus pseudocategory groups at each block revealed that discrimination ratios were higher in the category than in the pseudocategory ($P < 0.05$) at blocks 4–15; final discrimination ratios were also higher for the category than the pseudocategory group, indicating that even with maximum training birds in the pseudocategory group did not reach the same level of discrimination as the category group.

Number of stimuli discriminated

Figure 2b shows the number of S+ stimuli successfully discriminated from S– stimuli (see Methods for details on calculation) for the first 16 1000-trial blocks and on the final day of training by birds in the category and

pseudocategory groups. A sex \times discrimination group \times trial block mixed ANOVA revealed a main effect of block ($F(16, 208) = 11.36$, $P < 0.001$) but no main effects of sex ($F(1,13) = 1.70$, $P = 0.22$) or discrimination group ($F < 1$). There was a sex \times block interaction ($F(16,208) = 1.720$, $P = 0.045$) but no other interactions were significant ($F_s < 1$). We then looked at males and females separately, regardless of discrimination group. Males increased the number of S+s learned to one from block 3 ($m = 0.00$, $sd = 0.00$) to block 6 ($m = 1.65$, $sd = 1.92$), to two from block 6 to block 10 ($m = 2.12$, $sd = 2.1$), and to three or more from block 14 ($m = 1.75$, $sd = 2.05$) to the final day of training ($m = 3.13$, $sd = 1.81$). Females increased the number of S+s learned from block 3 ($m = 0.44$, $sd = 0.73$) to block 7 ($m = 1.67$, $sd = 1.88$), from block 7 to block 10 ($m = 3.00$, $sd = 1.80$), and from block 10 to the final day of training ($m = 4.33$, $sd = 0.71$). Females discriminated significantly more S+s than males only on trial block 16 ($t(15) = -1.86$, $P = 0.01$). Birds learned to discriminate their first S+ after about 3000 trials, males learned their second S+ 1000 trials faster than females (block 6 vs. block 7), females had learned more S+s than males by the end of 16,000 trials, but there was no sex difference by the final day of training.

Overall, discrimination ratio data indicated that birds in the category group learned to discriminate S+ from S– stimuli more quickly than birds in the pseudocategory group. Data related to the number of stimuli successfully discriminated were much more variable and therefore did not reveal a significant difference between birds in the two discrimination groups, but in the category group males knew more S+ stimuli after fewer trials than females; however, sexes and groups were not different by the end of training.

Discrimination of normal versus types of altered *fee* *bee* songs

Discrimination ratio

Figure 3 shows discrimination ratios for males (a) and females (b) in the category group over the first 16 1000-trial blocks and on the final day, separated by S– type. A sex \times stimulus type \times trial block mixed ANOVA revealed a main effect of stimulus type (Greenhouse Geisser-corrected $F(1.08, 10.76) = 156.30$, $P < 0.001$) and trial block ($F(16, 160) = 95.93$, $P < 0.001$) but not sex ($F(1,10) = 1.93$, $P = 0.20$). There was a significant stimulus \times block interaction (GG-corrected $F(3.13, 31.34) = 32.75$, $P < 0.001$) and a significant sex \times stimulus type \times block interaction (GG-corrected $F(4.14, 41.45) = 2.47$, $P < 0.001$) which led us to perform further analyses.

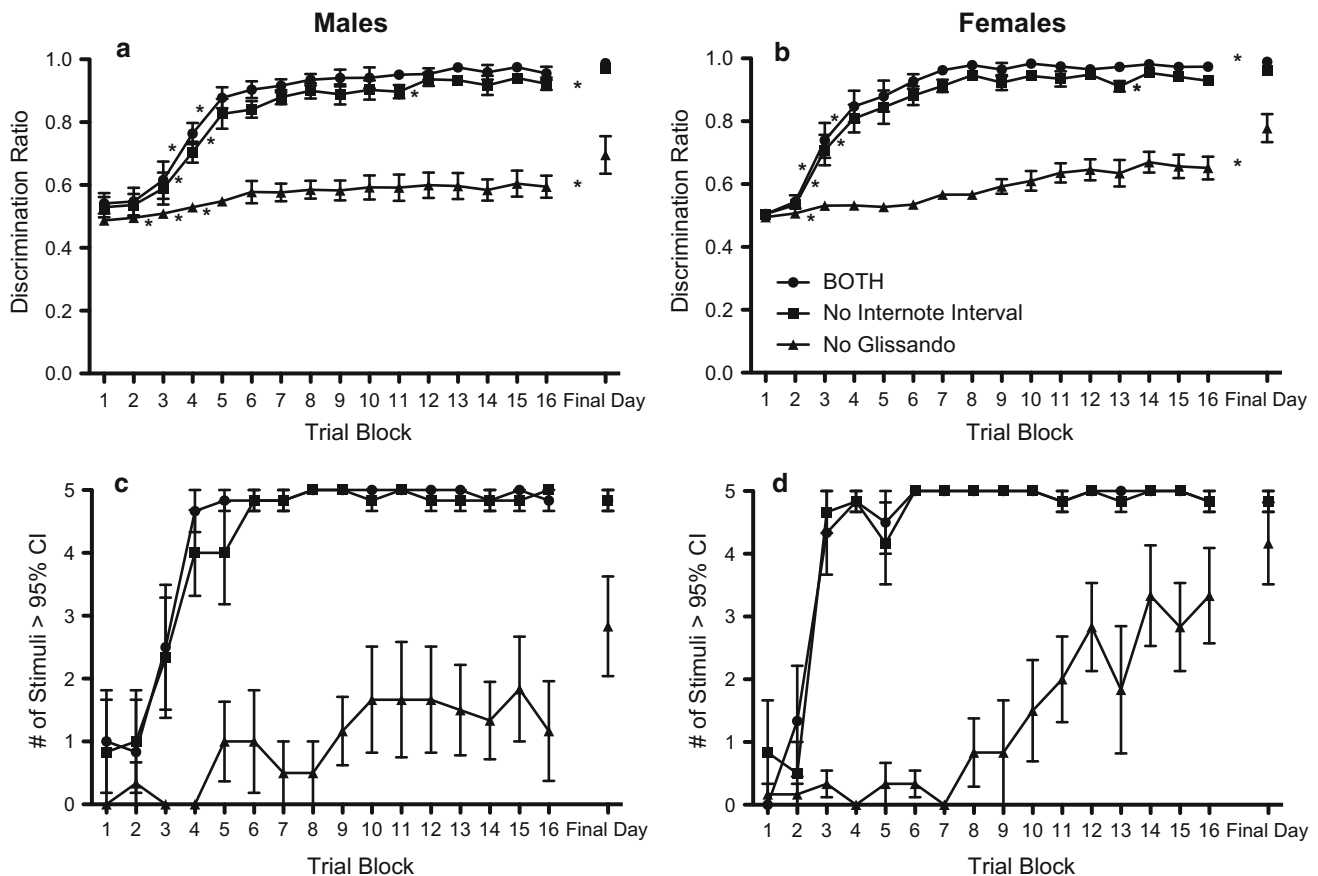


Fig. 3 Discrimination ratios (**a**, **b**) and number of stimuli successfully discriminated (**c**, **d**) of category group birds during discrimination training, broken down by type of altered S– stimulus. Error bars represent standard errors of the means. The discrimination ratios for

each of the three S–s were significantly different from each other from block 3 to the final block. Asterisks (*) indicate trial blocks in which discrimination ratio increased from one block to the next. ($P < 0.05$)

To examine the stimulus \times block interaction, we compared discrimination ratios among each S– stimulus types at each block using repeated measures ANOVAs and post hoc tests (LSD, $\alpha = 0.05$). There were no differences among discrimination ratios for the first two trial blocks (block 1 $F(2,22) = 3.42$, $P = 0.051$; block 2 $F(2,22) = 3.22$, $P = 0.06$), but there was for all blocks thereafter, including the final day of training ($F_s(2,22) 13.85 < 218.95$, $P < 0.001$). In all cases, the discrimination ratio was significantly higher when the S– had both alterations than when the S– lacked an inter-note interval, and both were significantly higher than when the S– lacked a *glissando*.

To describe more specifically any sex differences between the three S– discrimination tasks, we conducted a stimulus type \times trial block mixed ANOVA separately for each sex. For each sex, there was a significant main effect of stimulus type (male GG-corrected $F(1.04, 5.18) = 54.25$, $P = 0.001$; female $F(2,10) = 137.30$, $P < 0.001$) and trial block (male $F(16,80) = 44.86$, $P < 0.001$; female $F(16,80) = 52.66$, $P < 0.001$). There

was also a significant interaction between stimulus type and block (male GG-corrected $F(3.80, 18.996) = 20.06$, $P < 0.001$; female $F(32,160) = 15.77$, $P < 0.001$). We then compared discrimination ratios across trial blocks separately for each stimulus for both males and females (LSD, $\alpha = 0.05$). The most apparent differences were early in training. In the normal versus no *glissando* task, discrimination ratios for males increased significantly from block 2 to 3, from 3 to 4, and 4 to 5 and for females increased significantly from block 2 to 3, and from block 16 to 17. In the normal versus no inter-note interval task, discrimination ratios for males increased significantly from block 3 to 4, from 4 to 5, from 11 to 12, and from 16 to 17, and for females increased significantly from block 2 to 3, from 3 to 4, and from 13 to 14. In the normal versus both alterations task, the discrimination ratio increased significantly in males from block 3 to block 4 and from 4 to 5, and in females from block 2 to block 3, from 3 to 4, and from 16 to 17.

Finally, we examined the number of trial blocks required to reach criterion with respect to each S–

stimulus. A sex \times stimulus type ANOVA on blocks to criterion revealed a main effect of stimulus type ($F(1,10) = 5.50$, $P = 0.04$): birds reached criterion more quickly when the S– stimuli had both alterations than when only the inter-note interval was altered. There was no main effect of sex nor a sex \times stimulus type interaction.

Taken together, these results show that all birds, regardless of sex, performed the normal versus both alterations discrimination better than the normal versus no inter-note-interval discrimination, and the normal versus no inter-note-interval discrimination better than the no *glissando* discrimination. Females seemed to be slightly faster than males learning the S– discriminations when the inter-note interval was altered (BOTH and noINI), but males and females learned the discrimination when stimuli with no *glissando* was the S– at about the same rate.

Number of stimuli discriminated

Figure 3 also shows the number of S+s successfully discriminated by males (c) and females (d) in the category group over the first 16 1000-trial blocks and on the final day, separated by S– type. A sex \times stimulus type \times trial block mixed ANOVA revealed main effects of trial block ($F(16,160) = 34.227$, $P < 0.001$) and of stimulus type (Greenhouse–Geisser-corrected $F(1.02,10.20) = 89.26$, $P < 0.001$), with no main effect of sex ($F(1,10) = 1.07$, $P = 0.33$) or any interactions involving sex. There was a significant interaction between stimulus type and trial block (GG-corrected $F(4.96, 49.63) = 8.25$, $P < 0.001$), which prompted us to examine differences in learning across S– stimulus types using one-way ANOVAs and post hoc tests to compare number of S+s learned at each block. The number of S+s successfully discriminated did not differ based on S– type until block 3 ($F(2,22) = 22.74$, $P < 0.001$) when birds discriminated significantly more S+s when the S– had both alterations ($m = 3.42$, $sd = 2.19$) or only lacked the inter-note interval ($m = 3.5$, $sd = 2.07$) than when the S– only lacked the *glissando* ($m = 0.17$, $sd = 0.39$). This pattern continued over the remainder of acquisition and remained on the final day ($F(2,22) = 7.17$, $P < 0.005$): at each trial block, birds could discriminate more S+s when stimuli that lacked an inter-note interval (BOTH or noINI) were the S– than when stimuli that only lacked the *glissando* were the S–.

We then examined the number of S+s successfully discriminated compared to each type of S– separately using one-way repeated measures ANOVAs. When compared to S–s that lacked both the inter-note interval and *glissando* (BOTH; $F(16,176) = 34.50$, $P < 0.001$), birds increased the number of S+s discriminated between block 2 ($m = 1.08$, $sd = 2.02$) to block 3 ($m = 3.42$, $sd = 2.19$)

and block 3 to block 4 ($m = 4.75$, $sd = 0.62$). The pattern was similar when the S– lacked only the inter-note interval ($F(16,176) = 27.30$, $P < 0.001$), birds increased the number of S+s discriminated between block 2 ($m = 0.75$, $sd = 1.60$) to block 3 ($m = 3.5$, $sd = 2.07$) and block 3 to block 6 ($m = 4.92$, $sd = 0.29$). When the S+ lacked only the *glissando* ($F(16,176) = 8.54$, $P < 0.001$), the increase in the number of S+s discriminated was much slower, from block 4 ($m = 0$, $sd = 0$) to block 10 ($m = 1.58$, $sd = 1.93$) and from block 10 to the final day of training ($m = 3.5$, $sd = 1.83$), and never reached the same number as the other two S–s.

Generalization test

In order to further demonstrate whether birds were using categories to solve the discrimination rather than memorizing individual stimuli, we gave the category group birds new stimuli and tested whether birds treated these new S+ and S– stimuli as they did the S+ and S– stimuli from discrimination training. Figure 4 shows response rates to the novel S+ and S– stimuli compared those to the training S+ and S– that were presented during that same probe test. If learning was transferred to new stimuli, response rates to new S+ and S– stimuli should be high and low, respectively, similar to the already trained stimuli. A sex \times training phase (training vs. probe) \times contingency (S+ vs. S–) mixed ANOVA revealed a significant main effect of training phase ($F(1,10) = 8.04$, $P = 0.02$), with post hoc comparisons revealing that birds responded more to trained stimuli than to probe stimuli. There was also a significant main effect of contingency ($F(1,10) = 246.058$, $P < 0.001$), with birds responding more to trained and

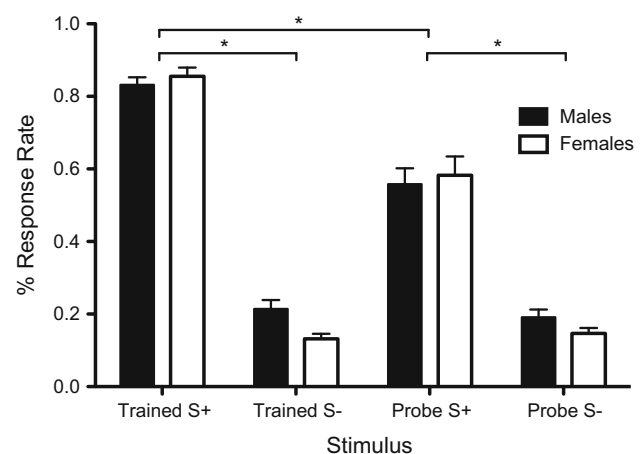


Fig. 4 Response rates during probe testing of male and female birds to previously trained S+ and S– and untrained S+ and S– presented during probe testing. Error bars represent standard errors of the means. Asterisks (*) indicate differences between specified bars at $P < 0.05$

probe S+ stimuli than to trained and probe S− stimuli. There was a significant interaction between phase and contingency ($F(1,10) = 12.80$, $P = 0.005$): birds responded more to trained S+s than to probe S+s, but there was no difference in responding between trained and probe S−s. In other words, birds transferred their training to previously untrained stimuli, although responding was not as strong to S+ probe stimuli as to training stimuli.

Discrimination of normal versus flat fee alone

Figure 5 shows the discrimination ratios over the first six 1000-trial blocks and the final day for individual pseudocategory birds on the second discrimination task of normal versus no *glissando* only. These birds completed an average of 10,800 trials ($sd = 2367.75$). Two females reached a final discrimination ratio of 0.80 or greater (but not criterion), and two birds never increased their discrimination ratio above chance levels. A sex \times trial block mixed ANOVA revealed a main effect of trial ($F(6,18) = 3.96$, $P = 0.01$), no main effect of sex ($F < 1$), nor an interaction ($F(6,18) = 2.39$, $P = 0.07$). Average (of males and females) discrimination ratios did increase from block 1 ($m = 0.48$, $sd = 0.04$) to block 3 ($m = 0.52$, $sd = 0.02$), and from block 4 ($m = 0.52$, $sd = 0.03$) to block 5 ($m = 0.54$, $sd = 0.04$); however, the average discrimination ratios for each trial block, including the final day of training, did not differ from chance when tested using a one-sample t test with 0.5 as the standard ($df = 4$, $P_s > 0.05$).

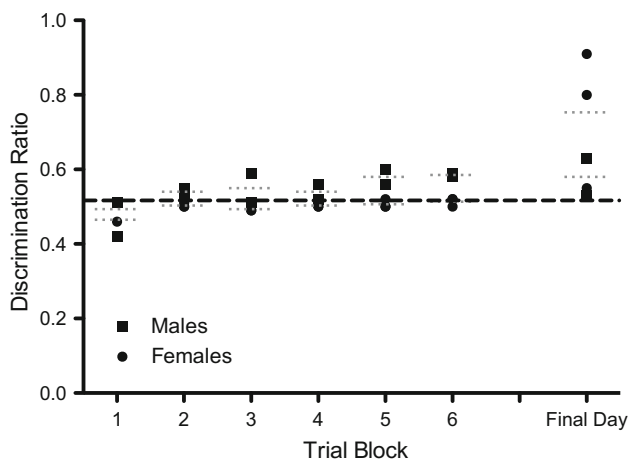


Fig. 5 Discrimination ratios for individual chickadees during a second training period given to pseudocategory birds ($N = 2$ males, 3 females) featuring exclusively normal (S+) and noGLIS (S−) stimuli. Grey dashed lines represent average discrimination ratios separately for males and females, and the black dashed line represents the discrimination ratio at chance

Discussion

The main objective of this study was to examine whether male and female black-capped chickadees could discriminate between normal *fee bee* songs and songs with altered species-typical pitch ratios associated with species recognition, and whether the type of alteration affected how quickly and accurately the birds learned the discriminations. Birds were able to use pitch ratio rules to categorize stimuli: birds who could use categories to discriminate normal from altered stimuli learned more quickly and achieved higher overall accuracy than birds who had to memorize individual stimuli. In addition, birds that learned categories based on alteration type were able to transfer their learning to novel stimuli of a different absolute frequency not presented during discrimination training.

We also wanted to determine if birds were more proficient at discriminating normal from altered songs depending on whether the *glissando*, the inter-note interval ratio, or both were eliminated. The data confirm our prediction that birds would better discriminate stimuli when the inter-note ratio was eliminated than when the *glissando* was eliminated: birds of both sexes learned to discriminate normal songs from songs without an inter-note interval (noINI, BOTH) better than songs lacking the *glissando* (noGLIS). What we did not expect was how poorly the birds discriminated the songs without the *glissando*, especially males who attend to this ratio alteration in the field. This discrimination was learned at a much slower rate than the other two discriminations, even when a group of birds were forced to attend to the flat *fee* stimuli by removing other altered stimuli in the stimulus pool. Finally, there appeared to be some subtle sex differences in the ability of birds to learn the discriminations: females were quicker than males at learning discrimination when S− stimuli lacked the inter-note interval (noINI and BOTH).

Glissando versus the inter-note interval discriminations

Although chickadees learned to discriminate between songs with typical ratios and songs with altered ratios, some alterations were easier to discriminate than others. Birds quickly learned to discriminate between normal songs and those with the inter-note interval removed, matching previous operant study findings showing that chickadees can discriminate between songs that differ with respect this ratio, whether increased, reduced, or eliminated (e.g. Weisman and Ratcliffe 1989; Weary and Weisman 1991; Hoeschele et al. 2012). Birds were even better at discriminating normal songs from songs with both the inter-note interval and the *glissando* removed (BOTH, ratio

from start to end of *fee* note AND ratio from end of *fee* to start of *bee* reduced to 1.00); perhaps the more aberrant a song is, the easier it is to discriminate from normal.

However, most birds had difficulty, more than we expected, discriminating normal songs from those with only the *glissando* eliminated: birds learned this task much slower than the other two, final discrimination ratios for normal versus n did not exceed 80%, and most birds never met overall criterion (3 days with a discrimination ratio over 0.90). This does not mean they could not perceive or discriminate the lack of *glissando*, but it was difficult to determine why those stimuli appeared to be learned more slowly in the context of the multi-S– category task.

One possibility was that although birds could perceive the lack of *glissando*, it was easier to perceive the lack of inter-note interval, and therefore birds attended to that cue over the lack of *glissando*. The relative ratio change is larger for the inter-note interval (1.134) than the *glissando* (1.056), so the degree to which the stimuli without an inter-note interval differ from normal song is greater than the difference between normal song and stimuli without a *glissando*. Interestingly, birds could discriminate the stimuli with both alterations better than stimuli with only the inter-note interval removed, meaning that the *glissando* did add some useable information for the discrimination task. However, the magnitude of this contribution, although significant, is not large: the difference in discrimination ratios based on stimuli with both alterations and those with only the inter-note interval altered is small, especially compared to how much lower discrimination ratios based on stimuli with only the *glissando* altered.

In an attempt to address the possibility that the chickadees were applying a hierarchy to the use of cues for species recognition, we presented chickadees that had previously completed the pseudocategory task with a simple category task: normal versus no *glissando* alone. This eliminated the possibility that birds could preferentially attend to stimuli that lacked the inter-note interval; if pseudocategory birds learned the simple normal versus no *glissando* task faster than category birds learned the normal versus no *glissando* task, this would provide evidence that perception of the two cues is equally accurate, but attention to the cues varies. However, the pseudocategory birds did not acquire this task any faster when forced to focus on S– that lacked the *glissando*. Although it is possible that previous training interfered with the acquisition of the normal versus no *glissando* task, birds would have been highly motivated to learn the task, and in the past have shown flexibility across successive tasks (e.g. reversal training Phillmore et al. 2002; McMillan et al. 2017).

Our results indicate, therefore, that the lack of *glissando* is extremely difficult to use as a discriminative cue and, even with repeated, focused exposure, birds appeared to

have difficulty perceiving the alteration. This was unexpected given that both males and females appear to attend to the *glissando* (Shackleton et al. 1992; Ratcliffe and Otter 1996). Our birds were in a quiet, controlled laboratory environment, so it is difficult to see how they would have had difficulty with hearing and perceiving this alteration, especially in comparison with birds in the field, where the signal-to-noise ratio would be much smaller, and in playback studies where the number of times a stimulus is heard is much fewer than in our study. However, Shackleton et al. (1992) themselves offer an explanation for lack of response to songs without a *glissando*: the alteration made the song sound as if it was coming from farther away. The persistence of the initial *fee* frequency is comparable with reverberation added to the original song from travel over distance. Therefore, the territorial males may not have responded because the song source appeared to be too far away to require a response (Naguib and Wiley 2001), compared to normal songs without the “additional” reverberation in the *fee*. Perhaps this is why birds in our study and Roach et al. (2016) did not respond to songs without a *glissando*.

Differences between findings in our laboratory and those of previous studies may stem from a variety of factors, but most obviously, the effects of captivity and laboratory experimentation may also have influenced behaviour, as has been documented in other songbirds (e.g. Dickens and Bentley 2014). However, our results are in fact consistent with recent work in our laboratory using the same normal and altered song stimuli as this study within a different experimental setup. Vocal response (number of vocalizations to playback) did not differ between normal stimuli and those lacking a *glissando*, but were lower in response to songs missing the inter-note interval (noGLIS and BOTH; Roach et al. 2016). This mirrors the differences in discriminative performance seen in the current study, in that birds did not seem to recognize when the *glissando* was missing, but did recognize the absence of the inter-note interval. They are also consistent with an operant study (Hahn et al. 2016) where chickadees could have used differences in the *glissando* to solve a discrimination task but did not seem to attend to them.

Sex differences in perception of alterations

Using the operant task allowed us to compare directly how male and female chickadees differ in their perception of and attention to species-specific ratios in the *fee bee* song. We expected females to perform better than males on discriminations between songs with normal ratios and songs with the inter-note interval eliminated. Although differences were subtle, discrimination ratios increased significantly 1000 trials earlier in females than in males

(see Fig. 2a), although these differences did not persist over training and males and females learned these discriminations to the same high level. We also predicted that males would be better than females at discriminating normal from songs with the *glissando* eliminated, but males and females were equally poor at acquiring this task. If anything, females outperformed males: the only bird to meet criterion when the S–s lacked only a *glissando* was a female, and in the analysis of birds that discriminated only normal from no *glissando* S–s, only females showed any block-to-block improvement. These findings partly match those of Hoeschele et al. (2012); females performed better than males at the end of a similar discrimination task. Although males in that study performed better than females at earlier stages of discrimination training, they did so only when discriminating among three species-atypical ratios (ratio of S+ 1.25; ratios of S– 1.19 and 1.31); in the current study, the rewarded (S+) stimuli within the category group always featured the species-typical ratio.

It may be that females have evolved better discriminative abilities due to the importance of detecting species-typical song ratio production when assessing male quality. Both dominant males and the females that mate with them experience greater reproductive success (Otter et al. 1998; Doucet et al. 2005), and dominant males are better than non-dominant males at maintaining the species-typical inter-note interval (Christie et al. 2004). If being able to accurately detect changes in this is an important contributor to sexual selection and eventual reproductive success, it may be that females have been forced to develop more finely tuned discriminative abilities, giving them the skill to outperform males on both ratio tasks. A recent study by our laboratory (Roach et al. 2016) examined neural responses to the same normal and altered song stimuli used in this study. We quantified immediate-early gene (*zenk* protein) activity in two brain areas associated with auditory perception and memory: caudomedial nidopallium (NCM) and caudomedial mesopallium (CMM), which respond preferentially to conspecific vocalizations. Although we did not find differences in response to normal compared to altered stimuli, indicating that this type of discrimination could occur elsewhere in the brain or in other cells not labelled, we did find that females had slightly but significantly more activity than males. This subtle sex difference in the brain could be linked to the subtle sex differences in performance.

Conclusion

The current study demonstrated that male and female black-capped chickadees can discriminate songs on the basis of the inter-note pitch interval with relative ease, but had considerable difficulty doing so based on the presence or absence

of the *glissando* within the *fee* note. In addition, we found that females were slightly better at learning the operant discrimination task. The next logical step for this research would be to examine whether there are differences in perceptual abilities when birds are photostimulated (in breeding condition) compared to when they are photorefractory (not in breeding condition). Females implanted with estradiol, mimicking the hormone levels found while in breeding condition, have more finely tuned selectivity in neural response in perceptual regions of the brain than birds without estradiol (Maney and Pinaud 2011). Therefore, comparing performance of females in breeding condition to those in non-breeding condition, and in turn to males, may clarify the degree to which females use inter-note interval in mate selection, and whether their ability to assess the ratio is enhanced in comparison with males. Most importantly, further exploring the importance of the two pitch ratios to male and female chickadees will shed light on our unclear picture of which pitch ratios are important not just for conspecific recognition but also transmitting information related to individual identity and quality. This would best be accomplished via studies that include both males and females, use biologically relevant response measures (e.g. a preference task in females), and, when possible, combine field and laboratory methodologies.

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Compliance with ethical standards

Conflict of interest All authors declare they have no conflict of interest.

Ethical approval All methods and procedures were approved by the University Committee on Lab Animals at Dalhousie in accordance with Canadian Council on Animal Care guidelines: Protocol # 12-023.

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